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(54) Title: PYRIMIDINE TARGETING HAIRPIN TRIPLEX-FORMING OLIGONUCLEOTIDES

(57) Abstract

    Hairpin triplex-forming oligonucleotides that target pyrimidine nucleic acids are disclosed. The oligonucleotides of the invention are characterized by having a duplex-forming region, a triplex-forming region, and a linker region wherein one of the internucleoside linkages between the duplex-forming and triplex-forming region is a 5'-5' or 3'-3' linkage. The duplex-forming region is comprised of purine nucleosides and has a sequence substantially complementary to a pyridine region of a target nucleic acid. The triplex-forming region is comprised of pyrimidine nucleosides and is substantially complementary in the Watson-Crick sense to the duplex-forming region. The linker region is comprised of nucleotides or other moieties that link the duplex- and triplex-forming regions. In the absence of target, the hairpin triplex-forming oligonucleotide folds back upon itself, the duplex- and triplex-forming regions running in parallel. In the presence of target nucleic acids, the duplex-forming region binds to the target by Watson-Crick bonding, and the triplex-forming region by Hoogsteen bonding, forming a triplex. The disclosed oligonucleotides display increased stability and are useful for modulating gene expression.

PYRIMIDINE TARGETING
HAIRPIN TRIPLEX-FORMING OLIGONUCLEOTIDES

BACKGROUND OF THE INVENTION

Field Of The Invention

5   The invention relates to synthetic oligonucleotides. More particularly,

the invention relates to synthetic oligonucleotides that are useful in gene

expression modulation.

Summary Of The Related Art

Since Zamecnik and Stephenson, *Proc. Natl. Acad. Sci. USA* 75, 280-

10   284 (1978) first demonstrated virus replication inhibition by synthetic

oligonucleotides, great interest has been generated in oligonucleotides as

therapeutic agents. In recent years the development of oligonucleotides as

therapeutic agents and as agents of gene expression modulation has gained

great momentum. The greatest development has been in the use of so-called

15   antisense oligonucleotides, which form Watson-Crick duplexes with target

mRNAs. Agrawal, *Trends in Biotechnology* 10, 152-158 (1992) extensively

reviews the development of antisense oligonucleotides as antiviral agents.

Also important, but somewhat less advanced, is the so-called antigene

oligonucleotide approach in which oligonucleotides form triplexes with target

20   DNA duplexes through Hoogsteen base pairing. Thoung and Helene, *Angew.*

Chem. Int. Ed. Engl. 32: 666-690 (1993) have recently reviewed developments

in this latter approach. *See also* Uhlmann and Peyman, *Chem Rev.* 90:543-584

(1990) and Chang and Pettitt, *Prog. Biophys. Mol. Biol.* 58:225-257 (1992).

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Triplex formation has been observed between DNA and various types of

oligonucleotides. Cooney et al., *Science* 241:456-459 (1988) teaches triplex

formation between DNA and an oligodeoxynucleotide phosphodiester. Latimer

et al., *Nucleic Acids Res.* **22**, 1549-1561 (1989) discloses triplex formation

5   involving oligodeoxynucleotide phosphorothioates. Kibler-Herzog et al.,

Nucleic Acids Res. **18**, 3545-3555 (1990) discloses triplex formation involving

short oligodeoxynucleotide methylphosphonates. Various base modifications

that enhance triplex formation are also known, including C5-methylation of

cytosine (Xodo et al., *J. Molec. Biol.* **19**, 5625-5631 (1991)), use of the

10   bicyclic cytosine analog, MODA (Young et al., *Proc. Natl. Acad. Sci. USA* **88**,

10023-10100 (1991)), and use of a synthetic α-anomeric nucleotide (Praseuth

et al., *Proc. Natl. Acad. Sci. USA* **85**, 1349-1353 (1988)). Giovannangeli et al.,

J. Am. Chem. Soc. **113**, 7775-7777 (1991) teaches that attachment of an

acridine intercalator onto the 5'-end of a capped oligonucleotide also enhances

15   triplex stability   Oligonucleotide-mediated triplex formation can cause

inhibition of transcription, at least *in vitro* (see Cooney et al. and Young et al.,

supra).

In recent years, several groups reported circular or foldback triplex

formation at homopurine single stranded sites using linear (Kandimalla and

20   Agrawal, *Gene* **149**, 115-121 (1994); Xodo et al., *Nucleic Acids Res.* **18**,

3557-3564 (1990); D'Souza et al., *Biomol. Struct. Dyn.* **10**, 141-152 (1992);

Noll et al., *Nucleosides Nucleotides* **13**, 997-1005 (1994)), circular (Kool, *J.*

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Am. Chem. Soc. **113**, 6265-6266 (1991); Prakash and Kool, *J. Am. Chem. Soc.*

114, 3523-3528 (1992); and D'Souza and Kool, *Bioorg. Med. Chem. Lett.* **4**,

965-970 (1994)), and ligand conjugated linear (Giovannangeli et al., *J. Am.*

Chem. Soc. **113**, 7775-7776 (1992); Giovannangeli, *Proc. Natl. Acad. Sci. USA*

5   **90**, 10013-10017 (1993); and Gryaznov and Lloyd, *Nucleic Acids Res.* **21**,

5909-5915 (1993)) homopyrimidine oligonucleotides, which may be useful as

agents for gene expression control at translation level by targeting mRNA. The

linear oligonucleotides that form foldback triplexes with homopyrimidine

strand were shown to disrupt guanine quadruplex structures if the target site

10   contains such quadruplex-forming base sequences. Kandimalla and Agrawal,

Nucleic Acids Res. **23**, 1068-1074 (1995). These oligonucleotides were

demonstrated to bind at oligopurine sites of a double helical DNA through

strand invasion mechanism by D-loop formation. Kandimalla et al. and

Manning, Agrawal, S. (in press).

15   Requirement of acidic pH conditions and limited base recognition are

impediments to full implementation of the triple helix approach. Several

modified bases (Ono et al., *J. Am. Chem. Soc.* **113**, 4032-4033 (1991);

Krawczyk et al., *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3761-3764 (1992); Miller

et al., *Biochemistry* **31**, 6788-6793 (1992); Koh et al., *J. Am. Chem. Soc.* **114**,

20   1470-1478 (1992); and Jetter and Hobbs, *Biochemistry* **32**, 3249-3254 (1993)

or alternate designs have been reported to overcome these problems, but with

limited success. Recently, an approach for targeting single stranded pyrimidine

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sites using circular oligonucleotides through G-G·C and T-A·T kind of triple

helix formati n has been proposed in which the third strand binds antiparallel

to the purine strand through reverse Hoogsteen hydrogen bonding, which is

weaker than Hoogsteen hydrogen bonded triplex. Wang and Kool, *J. Am.*

5   *Chem. Soc.* 116, 885 (1994).

Both the antisense and antigene oligonucleotide approaches have as

their goal gene expression modulation that is beneficial in understanding gene

expression and in therapeutic treatment of diseases or conditions involving

gene expression. Two major characteristics of oligonucleotide compounds that

10   are well suited to meet these goals are high specificity and an ability to interfere

with gene expression upon binding. Enhancement of these characteristics is

always desirable. There is, therefore, a need for new oligonucleotide

compounds having even greater specificity and more stable complex formation,

leading to increased ability to interfere with gene expression than existing

15   compounds.

BRIEF SUMMARY OF THE INVENTION

The invention provides novel antisense oligonucleotides for targeting

pyrimidine nucleic acid strands. These antisense oligonucleotides significantly

increase the number of sequences that can be targeted by triplex forming

20   oligonucleotides and also form more stable complexes with the target nucleic

acids.

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Oligonucleotides according to the invention have two complementary strands, which hybridize to each other and form a duplex structure. One strand of the duplex structure contains all purines and the other contains complementary all pyrimidine bases. These two strands are attached by either 3'-3' or 5'-5' linkage so that the complementary strands form a parallel-stranded hairpin-duplex. The two parallel strands are held together by Hoogsteen hydrogen bonding. We name this novel structure as parallel-stranded Hoogsteen duplex. The target sequence for such an antisense oligonucleotide will have the same sequence as that of the pyrimidine strand of the parallel-stranded duplex but in an opposite orientation, i.e., it will have opposite polarity. The purine strand of the antisense oligonucleotide will bind to the target pyrimidine strand in an antiparallel fashion through Watson-Crick hydrogen bonding resulting in a triplex structure (Fig. 3).

The complex formed between the target nucleic acid and oligonucleotides according to the invention has characteristics similar to both the duplex structure of the antisense approach and the triplex structure of the antigene approach to gene expression modulation. Because the same oligonucleotide performs both duplex and triplex-forming functions, this structure is called a hairpin triplex and oligonucleotides according to the invention, which complex with target nucleic acids to form a triplex, are called hairpin triplex-forming oligonucleotides.

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Hairpin triplex-forming oligonucleotides according to the invention are useful for carrying out *in vitro* studies of the kinetics of duplex and triplex formation under varying parameters. They are also useful in gene expression modulation studies, both *in vitro* and *in vivo*. The use of hairpin triplex-

5   forming oligonucleotides according to the invention provides an easily used alternative to the laborious method of modulating gene expression by deletion mutation. Accordingly, the hairpin triplex-forming oligonucleotides are useful research tools for the *in vitro* determination of the role of a targeted gene in biological processes. The importance of such a use can be appreciated when

10 one realizes that the elucidation of most known biological processes was accomplished by isolating and studying deletion mutants *in vitro* or in "knockout" animals.

The foregoing merely summarizes certain aspects of the present invention and is not intended, nor should it be construed, to limit the invention

15 in any manner. All patents and other publications cited herein establish the state of the art and are hereby incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show the antisense and antigene approaches to gene expression modulation.

20 Figure 2 shows C-G•C$^+$ and T-A•T base triplets with both Watson-Crick and Hoogsteen hydrogen bonding patterns implicated in triplex formation.

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Figure 3A diplays an example of how the complementary duplex- and triplex-forming regions of the hairpin triplex-forming oligonucleotides of the invention are oriented, and Figure 3B shows the orientation when an intramolecular duplex is formed.

Figure 4 A and B display triple-helix-forming constructs for targeting purine and pyrimidine single strands, respectively.

Figure 5 displays the derivative of the absorbance with respect to temperature for SEQ ID NOs 1-4 and 7. Oligonucleotide numbers without and with the "+" sign indicate the derivative of melting profiles of the oligonucleotide alone due to secondary structure, and in the presence of the DNA complementary strand, respectively.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention relates to synthetic oligonucleotides that are useful in gene expression modulation. The invention provides synthetic oligonucleotides that have greater specificity and more stable complex formation with target nucleic acids than existing oligonucleotides.

The antisense approach and the antigene approach, which are illustrated in Figure 1, are existing oligonucleotide-mediated approaches to gene expression modulation. In the antisense approach, the target nucleic acid is a single-stranded nucleic acid and the gene expression modulating agent is a complementary oligonucleotide. The complementary oligonucleotide and the target nucleic acid form a duplex by Watson-Crick base-pairing between the

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complementary bases. Modulation of gene expression presumably occurs by various mechanisms, including RNase H degradation of RNA, blocking of ribosome function on RNA, or blocking of enzyme function on RNA or DNA. In contrast, in the antigene approach, the target nucleic acid is a double-stranded nucleic acid and the gene expression modulating agent is an oligonucleotide that is capable of entering the major groove of the target duplex and forming Hoogsteen base pairs, such as those shown in Figure 2, with the purine strand of the target duplex. This interaction results in triplex formation. Gene expression modulation presumably occurs via interference with transcription.

Pyrimidine single strands can also be targeted by triple helix formation. This approach utilizes oligonucleotides having A·T and G·C base paired DNA hairpins in which the two strands are aligned in a parallel fashion for complexing with the DNA and RNA and pyrimidine single strands through triple helix formation Stable parallel stranded (ps) base pairing of A·T and G·C through Hoogsteen (Liu et al. *Biochem* 32, 11802-11809 (1993)) hydrogen bonding has been shown to be possible (Raghunathan et al., *Biopolymers* 34, 1573-1581 (1994)). The present inventiondemonstrates that these properties can be exploited to provide oligonucleotides that target pyrimidine single-stranded nucleic acids and form more stable complexes with the target than traditional antisense oligonucleotides.

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Oligonucleotides according to the invention have a duplex-forming region, a triplex-forming region, and a linker region. The duplex-forming region is complementary to the target nucleic acid and is able to hybridize with the target nucleic acid via Watson-Crick base-pairing, just as in the antisense

5   approach. The triplex-forming region is complementary in the Hoogsteen sense to the duplex-forming region but has opposite polarity, as depicted in Fig. 3A. That is, viewing the hairpin triplex-forming oligonucleotides of the invention as linear strands, when the sequence of one of the duplex- and triplex-forming regions is oriented $3'-5'$, the other is oriented $5'-3'$ and *vice versa*, as shown

10   in Fig. 3A. As used herein, the term "polarity" means the direction of the vector pointing from the 5' to 3' direction in the primary structure of the oligonucleotide. Accordingly, when an intramolecular duplex is formed, as depicted in Fig. 3B, the duplex- and triplex-forming regions are parallel in orientation. This is also the case when an intermolecular triplex is formed with

15   a target nucleic acid. As shown in Fig. 3B, Py_m and Pu_n, Py_{m-1} and Pu_{m-1}, . . ., Py_1 and Pu_1 are all Hoogsteen pairs. In this depiction, the number of nucleosides in the triplex-forming region (m) is equal to the number in the duplex-forming region (n), but this need not be the case. The duplex-forming region can have more nucleosides than the triplex-forming region.

20   The duplex-forming region of a hairpin triplex-forming oligonucleotide is characterized by having a nucleotide sequence that is sufficiently complementary to a target nucleic acid sequence to hybridize to the target

nucleic acid sequence under experimental or physiological conditions. Preferably, the duplex-forming region has from about 8 to 50 nucleotides, and most preferably has from about 12 to about 35 nucleotides. The target nucleic acid can have essentially any pyrimidine nucleotide sequence. For therapeutic,

5   medical, or experimental purposes, the duplex-forming region will preferably have a nucleotide sequence that is sufficiently complementary to hybridize under physiological or experimental conditions to the nucleotide sequence of the target nucleic acid that is involved in a particular disease state or physiological condition, or which is under investigation.

10   The duplex and triplex-forming regions are connected via a flexible linker or loop region, which allows the oligonucleotide to fold back upon itself and hybridize to form a Hoogsteen duplex (one in which the two strands are parallel). In the presence of a target pyrimidine nucleic acid, the duplex-forming region hybridizes in the normal Watson-Crick manner to form a duplex,

15   and the triplex-forming region remains in Hoogsteen base-pairing in the major groove of the duplex thus formed. The result of this interaction is the formation of a highly stable triplex. Such a complex (shown in Figure 4 for two oligonucleotides according to the invention and their targets) is called a triplex. Because oligonucleotides according to the invention are capable of forming

20   triplexes with target nucleic acids and form a hairpin within the oligonucleotide, such oligonucleotides are hereby denoted hairpin triplex-forming oligonucleotides.

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Oligonucleotides according to the invention have characteristics of both the antisense and antigene approaches. The target nucleic acid for oligonucleotides according to the invention is a single-stranded nucleic acid, as in the antisense approach, and the final complex is a triplex, as in the antigene approach.

The new constructs are shown in Fig. 4 and Table 1, *infra*. Fig. 4A depicts the strategy reported in Kandimalla and Agrawal, *supra*, Xodo et al., *supra*, D'Souza and Kool, *J. Biomol. Struct. Dyn. supra*, Noll et al., *supra*, and Prakash and Kool, *supra*. Fig. 4B depicts the new strategy described herein for targeting pyrimidine (C,T,U) strands. SEQ ID NOs 3 and 4 exist in parallel double helical hairpin structure under the experimental conditions (Liu et al., *supra*). In the presence of a single stranded pyrimidine target sequence that is complementary to the purine strand, however, oligonucleotides SEQ ID NOs 3 and 4 should form an antiparallel duplex via Watson-Crick hydrogen bonding. The pyrimidine strands of SEQ ID NOs 3 and 4 remain bound in the major groove of the antiparallel duplex in parallel fashion to the purine strand through Hoogsteen hydrogen bonding

As used herein, the term "complementary" means an oligonucleotide sequence that is sufficiently complementary (a) in the Watson-Crick sense to hybridize to a single stranded nucleic acid or (b) in the Hoogsteen base pairing sense to hybridize to a duplex DNA under the conditions (*e.g.*, temperature and pH) of interest. Unless otherwise indicated, as used herein the term hairpin

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triplex-forming oligonucleotide refers to the   ligonucleotides of the present

invention.

Hairpin triplex-forming oligonucleotides of the present invention differ

from previously disclosed foldback triplex-forming oligonucleotides in that in

5   the presently disclosed hairpin triplex-forming oligonucleotides the duplex-

forming region and the triplex-forming region have opposite polarity.

Furthermore, the presently disclosed oligonucleotides are designed to target

pyrimidine single strands. Therefore, the duplex-forming region is comprised

of all purine residues and is complementary in the Watson-Crick sense to the

10   target pyrimidine nucleic acid, thus facilitating hybridization to the target via

Watson-Crick base pairing in antiparallel fashion. The triplex-forming region

is a pyrimidine sequence, to complement the duplex-forming region, but in

parallel orientation.

The triplex-forming region preferably has at least about 8 nucleotides

15   and can be of any length up to the full length of the duplex-forming region. In

a preferred embodiment, the bases of the triplex-forming region include 5-

bromodeoxyuridine and/or 5-methylcytosine, each of which promote

Hoogsteen base pairing at or near physiological pH. The bicyclic cytosine

analog MODA, α-anomeric nucleotides and/or terminal acridines, other

20   terminal intercalators, or DNA cutting or modifying agents such as EDTA-FeII,

and cc-1065, or hydrophobic or amphophilic groups such as cholesterol,

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cyclodextrins, or polyamines may also be present in the triplex (or duplex)-forming region to promote triplex stability or target nucleic acid destruction.

The linker region of hairpin triplex-forming oligonucleotides is a flexible region that connects the duplex-forming region and the triplex-forming region. The linker region may be an oligonucleotide having from about 1 to about 10 nucleotides. In a preferred embodiment, the linker region is an oligonucleotide having about 5 nucleotides. When the linker region is comprised of nucleotides, either the duplex-forming region or the triplex-forming region are connected to the linker via a 5'-5' or 3'-3' internucleotide linkage, or the linker region has a 3'-3' or 5'-5' internucleotide linkage.

Alternatively, the linker region can be some other flexible chemical structure, such as a substituted or unsubstituted alkyl or aryl group having about 2 to 20 carbon atoms (e.g., isopropyl, o-xylyl), or ribose or 1, 2-dideoxyribose chains. In a preferred embodiment, the linker region is hexaethylene glycol. At a minimum, the linker region is a single covalent bond.

In the absence of target nucleic acid, the hairpin triplex-forming oligonucleotides form intramolecular duplexes, the triplex-forming region hybridizing to the complementary (in the Hoogsteen sense) duplex-forming region. Because the two-regions have opposite polarity, a parallel stranded duplex is formed, as opposed to the usual anti-parallel stranded duplex.

The fact that hairpin triplex-forming oligonucleotides do form both duplexes and triplexes is demonstrated by independent lines of evidence. First,

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under conditions that allow Hoogsteen base-pairing involving a cytosine-

containing triplex-forming strand (pH 5.0, under which conditions cytosine is

protonated), hairpin triplex-forming oligonucleotides form a complex with a

target nucleic acid that upon thermal denaturation yields a distinct increase in

5   A_{260} (each increase being indicative of a denaturation event). Fig. 5. The A_{260}

increase occurs at about the temperatures expected for thermal denaturation of

triplexes and duplexes. The higher temperature A_{260} increase takes place at the

expected temperature for disruption of a complex formed by both Watson-

Crick and Hoogsteen base-pairing (Kandimalla and Agrawal, *supra*). The

10   lower temperature A_{260} increase for duplexes of either antiparallel or parallel

nature would be expected for disruption of a less thermodynamically stable

complex involving only one of the base-pairing, Watson-Crick (anti-parallel)

or Hoogsteen (parallel), base pairings. In contrast, when conditions are altered

to prevent Hoogsteen base-pairing involving a cytosine-containing triplex-

15   forming strand (pH 7.4, under which conditions cytosine is not protonated),

only the lower temperature A_{260} increase is observed. Moreover, at

physiological concentrations of polyamines (1-3.0 mM spermine), which

stabilizes triplex formation, the higher A_{260} increase pattern is restored.

For the intact hairpin triplex-forming oligonucleotides and the various

20   structural regions referred to herein, except where explicitly stated otherwise,

structural features include, but are not limited to, ribonucleosides, 2'

substituted ribonucleosides, and/or deoxyribonucleotides. The internucleotide

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linkages may be a natural phosphodiester linkage or an artificial linkage, such

as, f r example, a phosphor thioate, phosphorodithioate, phosphoramidate,

alkylphosphonate, akylphosphonothioate, sulfonate, carbamate and/or

phosphotriester linkage.   Moreover, such oligonucleotides encompass

oligonucleotides having modifications on the bases and/or sugar residues as

well as those having nuclease-resistance conferring substituents or bulky

substituents at the 3′ and/or 5′ end.

Certain preferred embodiments of hairpin triplex-forming
oligonucleotides of the present invention are shown in Table 1.

TABLE I

Selected Foldback Triplex-Forming Oligos And An Antisense Control Oligo

Triplex Binding Region	Linker Region	Duplex Binding Region	SEQ ID NO
		5′-TCCTTCTTTCTTTTTT-3′	1*
		3′-TCCTTCTTTCTTTTTT-5′	2*
5′-AGGAAGAAAGAAAAAA-3′ — 3′-GGAGG	------	TTTTTTCTTTCTTCCT-5′	3†
3-AAAAAAGAAAGAAGGA-5 — 5-GGAGG	------	TCCTTCTTTCTTTTTT-3	4†
5-TCCTTCTTTCTTTTTT	------- CTCTC	------	TTTTTTCTTTCTTCCT-3	6*
5-TTTTTTCTTTCTTCCT	------- CTCTC	------	TCCTTCTTTCTTTTTT-3	7*

*Targeted to 5′-AGGAAGAAAGAAAAAA-3′ (SEQ ID NO 5)
†Targeted to SEQ ID NO 2

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Oligonucleotides SEQ ID NOs 3 and 4 showed high stability against exonucleases (snake venom or spleen phosphodiesterases), single- and double-strand specific endonucleases (S1 nuclease and DNase I), and 10% total fetal calf serum (non-heat inactivated) due to unusual 3'-3' or 5'-5' attachment, and the

5   formation of a stable duplex structure (Rippe and Jovine, *Biochemistry* 28:9542-9549 (1989), Tang and Temsamani and Agrawal, *Nucleic Acids Res.* 21:2729-2735 (1993)).

The new strategy described here for targeting single-stranded DNA and RNA pyrimidine sequences by formation of triple helices using ps hairpin

10   oligonucleotides not only significantly increases the number of sequences that can be targeted by triplex formation but also decreases the susceptibility of oligonucleotides to degradation by cellular nucleases either with or without chemical modification. The latter characteristic is significantly important in the development of therapeutic agents for *in vivo* uses and treatment of diseases.

15   Hairpin triplex-forming oligonucleotides according to the invention can be synthesized according to any of the procedures known in the art for oligonucleotide synthesis . *E.g.*, *Methods in Molecular Biology*, vol. 20, "Protocols for Oligonucleotides and Analogs: Synthesis and Properties" (Sudhir Agrawal, Ed., Humana Press, N.J., 1993). For example, U.S. Patent No.

20   5,047,524, the teachings of which are hereby incorporated by reference, teaches phosphoramidite synthesis of oligonucleotides. Alternatively, U.S. Patent No. 5,149,748, the teachings of which are hereby incorporated by reference, teaches

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an optimized H-phosph nate approach for oligonucleotide synthesis.   For

hairpin triplex-forming oligonucleotides containing non-oligonucleotide linker

regions, synthesis can still be carried out according to these procedures,

provided that any hydroxyl groups are first protected by an appropriate

protecting group, such as a dimethoxytrityl group, that any amino groups present

be protected by an appropriate protective group, such as a trifluoroacetyl group,

and that one end be linked to an appropriate coupling group, such as a β-

cyanoethyl-phosphoramidite or H-phosphonate group.

Hairpin triplex-forming oligonucleotides according to the invention are

useful for a variety of purposes. First, they are useful for *in vitro* studies of

nucleic acid triplex formation. In a hairpin triplex-forming oligonucleotide it is

possible to vary many parameters, such as internucleotide linkage types, base

modifications, linker length and flexibility, etc. to study the kinetics of triplex

formation and disruption, which may be a biologically important process.

In addition, hairpin triplex-forming oligonucleotides can be used in place

of traditional antisense oligonucleotides in tissue culture and animal models for

studying gene expression. In these systems, the increased specificity and

complex stability of hairpin triplex-forming oligonucleotides is beneficial. The

hairpin triplex forming olgionucleotides according to the invention provide an

attractive alternative to deletion mutation in the modulation of gene expression

for the *in vitro* or *in vivo* determination of the role of the targeted gene in

biological processes.

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Finally, hairpin triplex-forming oligonucleotides are useful as therapeutic

agents for diseases or physiological conditions involving expression of specific

genes.   The disease or condition that a particular hairpin triplex-forming

oligonucleotide is useful for treating will depend upon the nucleotide sequence

5   to which the duplex-forming region is sufficiently complementary to hybridize

under physiological conditions. In many cases the nucleic acid sequence will be

a virus nucleic acid sequence. The use of antisense oligonucleotides to inhibit

various viruses is well known, and has recently been reviewed in Agrawal,

Tibtech 10, 152-158 (1992). The lessons learned from antisense antivirals can

10   be applied to duplex-forming regions. Viral nucleic acid sequences that

hybridize to effective antisense oligonucleotides have been described for many

viruses, including human immunodeficiency virus type 1 (U.S. Patent No.

4,806,463, the teachings of which are herein incorporated by reference), Herpes

simplex virus (U.S. patent No. 4,689,320, the teachings of which are hereby

15   incorporated by reference), Influenza virus (U.S. Patent No. 5,194,428, the

teachings of which are hereby incorporated by reference), and Human papilloma

virus (Storey et al., *Nucleic Acids Res.* 19, 4109-4114 (1991)). Sequences

hybridizing to any of these nucleic acid sequences can be used for the duplex-

forming region of hairpin triplex-forming oligonucleotides, as can nucleotide

20   sequences complementary to nucleic acid sequences from any other virus.

Additional viruses that have known nucleic acid sequences against which hairpin

triplex-forming oligonucleotides can be prepared include, but are not limited to,

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Foot and Mouth Disease Virus (*See* Robertson et al., *J. Virology* 54, 651 (1985);

Harris et al., *J. Virology* 36, 659 (1980)), Yellow Fever Virus (*See* Rice et al.,

Science 229, 726 (1985)), Varicella-Zoster Virus (*See* Davison and Scott, J.

Gen. Virology 67, 2279 (1986), Cucumber Mosaic Virus (*See* Richards et al.,

5   *Virology* 89, 395 (1978)), Hepatitis B Virus (*See* Raney and McLachlen, in

Molecular Biology of Hepatitis B Virus (CRC Press, 1991)), Hepatitis C Virus

(*See* Miller and Purcell, *Proc. Natl. Acad. Sci. USA* 87, 2057-2061 (1990); *Proc.*

Natl. Acad. Sci. USA 89, 4942-4946 (1992); *J. General Virology* 74, 661-668

(1993)), Human Papilloma Virus (Storey et al., *supra*), and Respitory Syncitial

10   Virus (*See* Collins, in *The Paramyxo Viruses*, Chapter 4, pp. 103-162 (David W.

Kingsbury, Ed., 1991)). Alternatively, the duplex-forming region can have a

nucleotide sequence complementary to a nucleic acid sequence of a pathogenic

organism. The nucleic acid sequences of many pathogenic organisms have been

described, including the malaria organism, *Plasmodium falciparum*, and many

15   pathogenic bacteria Nucleotide sequences hybridizing to nucleic acid sequences

from any such pathogenic organism can form the duplex-forming region of

hairpin triplex-forming oligonucleotides. Examples of pathogenic eukaryotes

having known nucleic acid sequences against which hairpin triplex-forming

oligonucleotides can be prepared include, but are not limited to *Trypanosoma*

20   *brucei gambiense* and *Leishmania* (*See* Campbell et al., *Nature* 311, 350

(1984)), *Fasciola hepatica* (*See* Zurita et al., *Proc. Natl. Acad. Sci. USA* 84,

2340 (1987). Antifungal hairpin triplex-forming oligonucleotides can be

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prepared using a duplex-forming region having a nucleotide sequence that is complementary to a nucleic acid sequence from, *e.g.*, the chitin synthetase gene, and antibacterial hairpin triplex-forming oligonucleotides can be prepared using, *e.g.*, the alanine racemase gene. In yet another embodiment, the duplex-forming

5   region of hairpin triplex-forming oligonucleotides can have a nucleotide sequence complementary to a cellular gene or gene transcript, the abnormal expression or product of which results in a disease state. The nucleic acid sequences of several such cellular genes have been described, including prion protein (Stahl and Prusiner, *FASEB J.* 5, 2799-2807 (1991)), the amyloid-like

10   protein associated with Alzheimer's disease (U.S. Patent No. 5,015,570), and various well-known oncogenes and proto-oncogenes, such as c-*myb*, c-*myc*, c-*abl*, and n-*ras*. In addition, oligonucleotides that inhibit the synthesis of structural proteins or enzymes involved largely or exclusively in spermatogenesis, sperm motility, the binding of the sperm to the egg or any other

15   step affecting sperm viability may be used as contraceptives for men. Similarly, contraceptives for women may be oligonucleotides that inhibit proteins or enzymes involved in ovulation, fertilization, implantation or in the biosynthesis of hormones involved in those processes. Hypertension can be controlled by oligonucleotides that suppress the synthesis of angiotensin converting enzyme

20   or related enzymes in the renin/angiotensin system; platelet aggregation can be controlled by suppression of the synthesis of enzymes necessary for the synthesis of thromboxane A2 for use in myocardial and cerebral circulatory disorders,

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infarcts, arteriosclerosis, embolism and thrombosis; deposition of cholesterol in

arterial wall can be inhibited by suppression of the synthesis of fatty acyl co-

enzyme A: cholesterol acyl transferase in arteriosclerosis; inhibition of the

synthesis of cholinephosphotransferase may be useful in hypolipidemia. There

5   are numerous neural disorders in which hairpin triplex-forming oligonucleotides

can be used to reduce or eliminate adverse effects of the disorder. For example,

suppression of the synthesis of monoamine oxidase can be used in Parkinson's

disease; suppression of catechol O-methyl transferase can be used to treat

depression; and suppression of indole N-methyl transferase can be used in

10   treating schizophrenia. Suppression of selected enzymes in the arachidonic acid

cascade which leads to prostaglandins and leukotrienes may be useful in the

control of platelet aggregation, allergy, inflammation, pain and asthma.

Suppression of the protein expressed by the multi-drug resistance (*mdr*) gene,

which is responsible for development of resistance to a variety of anti-cancer

15   drugs and is a major impediment in chemotherapy may prove to be beneficial in

the treatment of cancer. Nucleotide sequences complementary to nucleic acid

sequences from any of these genes can be used for the duplex-forming region of

hairpin triplex-forming oligonucleotides according to the invention, as can be

oligonucleotide sequences complementary to any other cellular gene or gene

20   transcript, the abnormal expression or product of which results in a disease state.

Antisense regulation of gene expression in plant cells has been described in U.S.

Patent No. 5,107,065. Since the nucleotide sequence of the duplex-forming

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region can be adapted to form Watson-Crick base pairs with essentially any gene, the therapeutic spectrum   f hairpin triplex-forming oligonucleotides sh_uld be very broad. Still, certain diseases are of particular interest. For example, a variety of viral diseases may be treated by hairpin triplex-forming oligonucleotides, including AIDS, ARC, oral or genital herpes, papilloma warts, flu, foot and mouth disease, yellow fever, chicken pox, shingles, HTLV-leukemia, and hepatitis. Among fungal diseases treatable by hairpin triplex-forming oligonucleotides are candidiasis, histoplasmosis, cryptococcocis, blastomycosis, aspergillosis, sporotrichosis, chromomycosis, dematophytosis and coccidioidomycosis. The method can also be used to treat rickettsial diseases (e.g., typhus, Rocky Mountain spotted fever), as well as sexually transmitted diseases caused by *Chlamydia trachomatis* or *Lymphogranuloma venereum*. A variety of parasitic diseases can be treated by hairpin triplex-forming oligonucleotides including amebiasis, Chegas' disease, toxoplasmosis, pneumocystosis, giardiasis, cryptosporidiosis, trichomoniasis, and *Pneumocystis carini* pneumonia, also worm (helminthic diseases) such as ascariasis, filariasis, trichinosis, schistosomiasis and nematode or cestode infections. Malaria can be treated by hairpin triplex-forming oligonucleotides regardless of whether it is caused by *P. falciparum*, *P. vivax*, *P. orale*, or *P. malariae*. The infectious diseases identified above can all be treated by hairpin triplex-forming oligonucleotides because the infectious agents for these diseases are known and thus hairpin triplex-forming oligonucleotides according to the invention can be

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prepared, having a target-forming region that has a nucleotide sequence that

hybridizes to a nucleic acid sequence that is an essential nucleic acid sequence

for the propagation of the infectious agent, such as an essential gene.

Because DNA can breathe (*see, e.g.,* Ussery and Sindem, *Biochemistry*

5   *32*, 6206 (1993)), hairpin triplex-forming oligonucleotides can form triplexes

with DNA. *See, e.g.,* Hélène and Toulmé, *Biochimica et Biophysica Acta* **1049**,

99 (1990) at page 100.

Cancer cells have a much lower pH than normal cells. *See, e.g.,* Lutz F.

Tietze in *Molecular Aspects of Chemotherapy: Proceedings of the Second*

10   *International Symposium on Molecular Aspects of Chemotherapy,* Chapter 5 (E.

Borowski and D. Shugar, Eds., Pergamon Press, 1990). Accordingly, one can

design hairpin triplex-forming oligonucleotides that are capable of forming

hairpin triplexes in cancer cells preferentially. Oligonucleotide activity in cancer

cells can be obtained by designing hairpin triplex-forming oligonucleotides to

15   contain more cytosines than thymine, because cytosine must be protonated,

preferably at low pH, to form Hoogsteen bonds. It is this difference that

provides the opportunity to design hairpin triplex-forming oligonucleotides that

preferentially form hairpin triplexes in cancer cells only. For normal cells,

methylcytosines, which can be protonated near physiological pH (6.5-6.8), can

20   be used.

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The following examples are intended to further illustrate certain preferred

embodiments of the invention and are not intended, nor should they be

construed, to limit the invention in any manner.

EXAMPLES

Example 1

5

Synthesis of 5'-5' and 3'-3' Oligonucleotides

The 5'-5' attached oligonucleotides were synthesized on normal solid

support (CPG) on which the new oligonucleotide grows in the 3'-5' direction

using normal 5'-DMTr 3'-β-cyanoethylphophoramidites (Millipore, Burlington,

10   MA). At the 5'-5' attachment site, 3'-phosphoramidites were replaced with 3'-

DMTr- 5'-β-cyanoethylphosphoramidites (Glen Research, Sterling, VA) and

synthesis was continued to obtain the desired oligonucleotide.

The 3'-3' attached oligonucleotide synthesis was carried out on 5'-

attached solid support (Glen Research) using 3'-DMTr-5'-β-

15   cyanoethylphosphoramidites From the 3'-3' attachment site, synthesis was

continued with normal monomer synthons. van de Sande et al., Science 241,

551 (1988).

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Example 2

Thermal Denaturation Studies

Each antisense oligonucleotide (1.2 µM) was mixed with equal amount

of target in a buffer of 100 mM sodium acetate, pH 5.0, containing 10 mM

5   magnesium chloride, heated to 95°C for 10 min, allowed to come to room

temperature slowly and then left at 4°C overnight. The melting temperature of

the each sample was measured on a Perkin-Elmer Lambda 2 spectrophotometer

attached to a thermalcontroller. Melting curves were recorded by measuring

absorbance at 260 nm as a function of temperature at a heating rate of

10   0.5°C/min. Melting transition mid-points were determined by plotting dA/dT v.

T. Each value is an average of two independent experiments. Uncertainties in

the T°C values is about 1.0°C The results are displayed in Figure 5 and Table

2. In the RNA sequence, U replaces T. The numbers in parentheses are ΔT_m

values relative to the Watson-Crick aps duplex of oligonucleotide SEQ ID NO

15   2. Triple helices of oligonucleotide SEQ ID NOs 6 and 7 with RNA purine

target are not stable and, therefore, are not shown.

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TABLE 2
Oligonucleotide Sequences and Thermal Melting Data

| SEQ ID NO. | T_m (°C) | | | |
| | pH 5.0 | | pH 7.6 | |
	DNA	RNA	DNA	RNA
1+5	41.0	---	45.3	---
2+5	48.6	32.7	52.0	32.1
3	47.8	---	48.6	---
4	45.3	---	46.9	---
3+2	63.1 (14.5)	53.3 (20.6)	54.6 (2.6)	41.4 (9.3)
4+2	63.4 (14.8)	53.7 (21.0)	53.3 (1.3)	43.1 (11.0)
6+5	62.5 (13.9)	---	54.2 (2.2)	(2.2)
7+5	63.3 (14.7)	---	54.8 (2.8)	---

In these UV thermal melting studies, oligonucleotides SEQ ID NOs 3 and 4 showed T_ms of 47 8°C and 45.3°C, respectively, due to the formation of ps duplexes. CD studies confirmed formation of Hoogsteen hydrogen bonding ps hairpin duplexes (Liu et al. *supra*) below pH 6.0 by SEQ ID NOs 3 and 4. Native polyacrylamide gel electrophoresis experiments revealed the difference between ps and aps (anti-parallel strand) hairpin duplexes (Liu et al., *supra*). The linear ps and aps duplexes formed by control oligonucleotides SEQ ID NOs 1 and 2

- 27 -

with the c mplementary strand have T_ms of 41.0 and 48.6°C, respectively (Table 2).

In the presence of the DNA target strand SEQ ID NO 2, oligonucleotides SEQ ID NOs 3 and 4 formed stable triplexes with considerably higher stability than the ps or aps duplexes. The resulting triplexes showed single, cooperative melting transitions with T_ms of 63.1 and 63.4°C (pH 5.0) for SEQ ID NOs 3 and 4, respectively. Both oligonucleotides formed complexes with 1:1 stoichiometry as determined by electrophoretic mobility shift assay on native polyacrylamide gels, similar to the triplexes formed with control oligonucleotides SEQ ID NOs 6 and 7 Kandimalla and Agrawal, *Gene, supra*. The triplexes of SEQ ID NOs 6 and 7 with their single-stranded DNA purine target SEQ ID NO 2 showed T_ms of 62.5 and 63.3°C, respectively. Oligonucleotides SEQ ID NOs 3 and 4 formed triplexes at pH 7.6 in the presence of spermine (Moser and Dervan, *Science* 238:645 (1987) and Harvey et al. *Antisense Res. Dev.* 1:307 (1991)), although the triplexes are 10°C less stable than those formed at pH 5.0 (Povsic et al. *J. Am. Chem. Soc.* 111:3059-3061 (1989); Xodo et al. *Nucleic Acids Res.* 19:5625-5631 (1991)) Oligonucleotides containing cytosines require acidic pH conditions for triplex formation. Triplex formation at physiological pH is achieved in the presence of 1 mM spermine. The triplex formed at neutral pH in the presence of spermine is less stable, however, than the triplex formed at acidic pH conditions. Alternately, 5-methylcytosines can be used in order to promote triplex formation at neutral pH without using spermine. Similar results were

obtained with oligonucleotides SEQ ID NOs 6 and 7 at pH 7.6. Comparison of the melting profiles and CD spectra of the triplexes SEQ ID NOs 3 and 4 to those of SEQ ID NOs 6 and 7 revealed similar characteristics, suggesting the formation of highly stable triplexes in both the cases (Kandimalla and Agrawal, *Gene*

5   149:115-121, Prakash and Kool, *J. Amer. Chem. Soc.* 114:3523-3528 (1992).

Oligonucleotides SEQ ID NOs 3 and 4 exhibited strong binding to the single-stranded RNA target SEQ ID NO 2 (with T_ms of 53.3°C and 53.7°C at pH 5.0. Under the same experimental conditions, the heteroduplex of RNA SEQ ID NO 2 and its complementary strand showed a T_m of 32.7°C (Roberts and

10   Crothers, *Science* 258:1463-1466 (1992). Triple helices of SEQ ID NOs 3 and 4 with RNA target sequence have 20°C (ΔT_m) higher thermal stability over the Watson-Crick heteroduplex. The ΔT_m calculated for RNA triplex is about 6°C higher than ΔT_m calculated for DNA triplex (Table 2). This difference in ΔT_m is about 10°C higher for RNA triplex at pH 7.6 than at pH 5.0 (Table 2)

15   suggesting a more stable triplex formation with RNA target at physiological pH. Recent studies suggest that triple helix types of oligonucleotides SEQ ID NOs 6 and 7 with RNA purine target strand are not stable (Kandimalla and Agrawal, *Gene* 149:115-121 (1994). Kandimalla and Agrawal *Nucleic Acids Res.* 23:1068-1074 (1995), Roberts and Crothers, *Science* 258:1463-1466 (1992), Han and

20   Dervan, *Proc. Natl. Acad. Sci. USA*, 90: 3806-3810 (1993), Escude et al., *Nucleic Acids Res.* 22:5547-5553 (1993), Wang and Kool, *Nucleic Acids Res.* 22:2326-2333 (1994)). Further studies of the formation of triple helices under

- 29 -

different temperature and pH conditions revealed stable triplex formation even when the ps hairpin SEQ ID NOs 3 and 4 and target DNA or RNA strand SEQ ID NO 2 were incubated at 4°C confirming that the triplex forms readily and does not require conformational changes.

- 30 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Kandimalla, Ekambar R.
                Agrawal, Sudhir

    (ii) TITLE OF INVENTION: Pyrimidine-Targeting Hairpin
          Triplex-Forming Oligonucleotides

   (iii) NUMBER OF SEQUENCES: 7

    (iv) CORRESPONDENCE ADDRESS:
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        (B) STREET: 10 S. Wacker Drive Suite 3000
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        (D) STATE: Illinois
        (E) COUNTRY: U.S.A.
        (F) ZIP: 60606

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version
    #1.25

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

   (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: Greenfield, Michael S.
        (B) REGISTRATION NUMBER: 37,142
        (C) REFERENCE/DOCKET NUMBER: 95,550

    (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: (312)715-1000
        (B) TELEFAX: (312)715-1234

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 16 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (iii) HYPOTHETICAL: NO

- 31 -

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCCTTCTTTC TTTTTT   16

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 16 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTTTTTCTTT CTTCCT   16

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 37 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(ix) FEATURE:
        (A) NAME/KEY: misc_feature
        (B) LOCATION: 17..37
        (D) OTHER INFORMATION: /note= "nucleotides
           17-37 are listed in the 3'->5' direction".

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGGAAGAAAG AAAAAAGGAG GTTTTTTCTT TCTTCCT   37

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 37 base pairs

- 32 -

    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(ix) FEATURE:
    (A) NAME/KEY: misc_feature
    (B) LOCATION: 1..16
    (D) OTHER INFORMATION: /note= "nucleotides
       1-16 are listed in the 3'->5' direction"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AAAAAAGAAA GAAGGAGGAG GTCCTTCTTT CTTTTTT   37

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 16 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGGAAGAAAG AAAAAA   16

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 37 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TCCTTCTTTC TTTTTTCTCT CTTTTTTCTT TCTTCCT   37

- 33 -

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 37 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: YES

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTTTTCTTT CTTCCTCTCT CTCCTTCTTT CTTTTTT   37

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We claim:

1.   A hairpin triplex-forming oligonucleotide comprising a duplex-forming

region, a triplex-forming region, and a linker region, wherein the duplex-forming

region is complementary to a pyrimidine region of a target nucleic acid and is

5   able to hybridize to that region and thereby form a duplex, and wherein the

triplex-forming region is complementary to the duplex-forming region and is able

to hybridize to both the duplex-forming region, thereby-forming a hairpin

duplex, as well as to the duplex formed between the duplex-forming region and

the pyrimidine region of the target nucleic acid, thereby-forming a triplex, and

10   wherein the duplex-forming region and triplex-forming region have opposite

polarity and are connected to each other by the linker region.

2.   An oligonucleotide according to claim 1 wherein the linker is a non-

nucleotide linker.

3.   An oligonucleotide according to claim 2 wherein the linker is

15   hexaethylene glycol

4.   An oligonucleotide according to claim 1 wherein the linker region

comprises from 3 to about 10 nucleotides in length and one of the linkages

between the duplex-forming region and the triplex-forming region is a 3'-3' or

5'-5' internucleoside linkage.

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5.   An oligonucleotide acc rding to claim 4 wherein the linker is about 5
nucleotides in length.

6.   An oligonucleotide according to claim 4 wherein the duplex-forming
region is from about 8 to about 50 nucleotides in length.

5   7.   An oligonucleotide according to claim 5 wherein the duplex-forming
region is from about 25 to 35 nucleotides in length.

8.   An oligonucleotide having sequence 5'-AGGAAGAAAGAAAAAA-3'-
3'-GGAGGTTTTTTCTTTCTTCCT-5' (SEQ ID NO 3).

9.   An oligonucleotide having sequence 3'-AAAAAAGAAAGAAGGA-5'-
10   5'-GGAGGTCCTTCTTTCTTTTTT-3' (SEQ ID NO 4).

10.   A method of modulating the expression of a nucleic acid having a
homopyridine sequence, the method comprising contacting the nucleic acid with
a hairpin triplex-forming oligonucleotide according to Claim 1.

11.   A method of modulating the expression of a nucleic acid having a
15   homopyridine sequence, the method comprising contacting the nucleic acid with
a hairpin triplex-forming oligonucleotide according to Claim 2.

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12.   A method of modulating the expression of a nucleic acid having a homopyridine sequence, the method comprising contacting the nucleic acid with a hairpin triplex-forming oligonucleotide according to Claim 3.

13.   A method of modulating the expression of a nucleic acid having a homopyridine sequence, the method comprising contacting the nucleic acid with a hairpin triplex-forming oligonucleotide according to Claim 4.

14.   A method of modulating the expression of a nucleic acid having a homopyridine sequence, the method comprising contacting the nucleic acid with a hairpin triplex-forming oligonucleotide according to Claim 5.

15.   A method of modulating the expression of a nucleic acid having a homopyridine sequence, the method comprising contacting the nucleic acid with a hairpin triplex-forming oligonucleotide according to Claim 6.

16.   A method of modulating the expression of a nucleic acid having a homopyridine sequence, the method comprising contacting the nucleic acid with a hairpin triplex-forming oligonucleotide according to Claim 7.
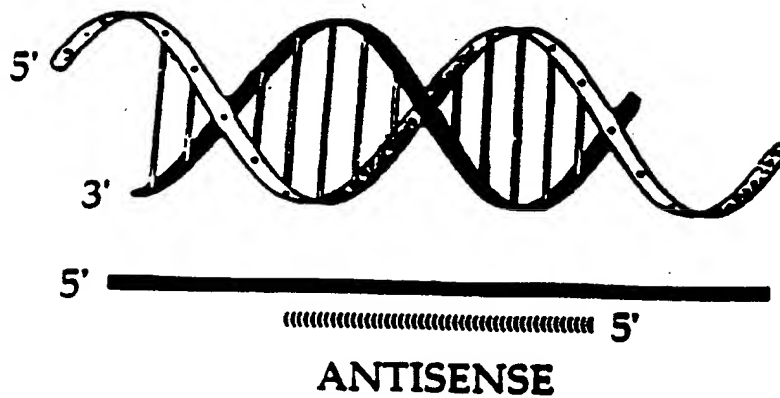
17.   A method of modulating the expression of a nucleic acid having a homopyridine sequence, the method comprising contacting the nucleic acid with a hairpin triplex-forming oligonucleotide according to Claim 8.

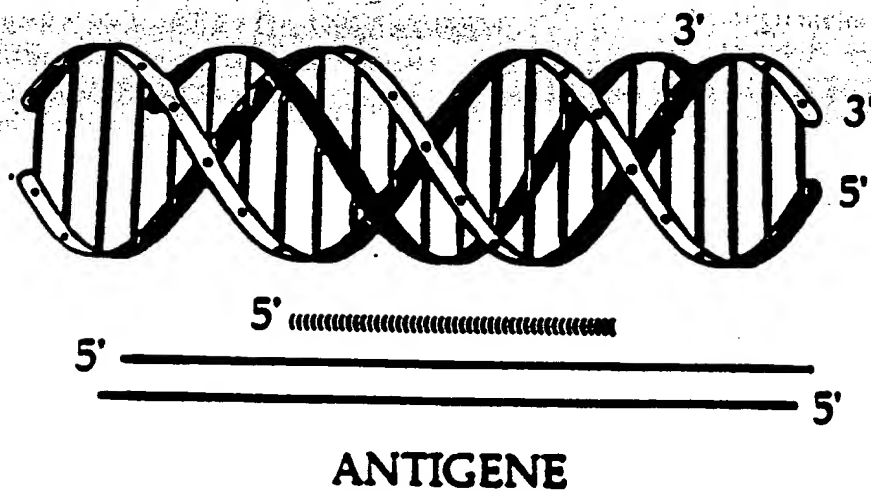18.   A method of modulating the expression of a nucleic acid having a homopyridine sequence, the method comprising contacting the nucleic acid with a hairpin triplex-forming oligonucleotide according to Claim 9.
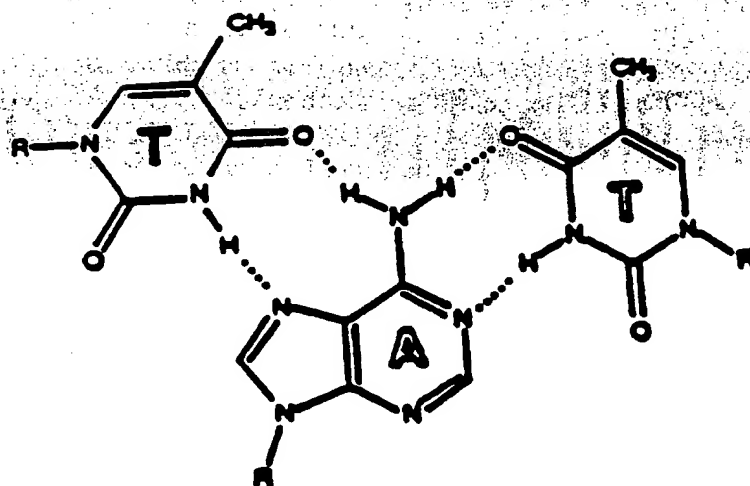
ANTISENSE

FIG. 1A

ANTIGENE

FIG. 1B

FIG. 2

Duplex-forming Region   Triplex-forming Region

5'-Pu$_1$-Pu$_2$-Pu$_3$- . . . -Pu$_{n-2}$-Pu$_{n-1}$-Pu$_n$-3' —Linker—3'-Py$_m$Py$_{m-1}$Py$_{m-2}$- . . .-Py$_3$-Py$_2$-Py$_1$-5'

5' ——————————→ 3'   3' ←—————————— 5'

A

Linker

3'   3'

Pu$_n$ ● Py$_m$

Pu$_{n-1}$ ● Py$_{m-1}$

Pu$_{n-2}$ ● Py$_{m-2}$

.   .

.   .

.   .

Pu$_3$ ● Py$_3$

Pu$_2$ ● Py$_2$

Pu$_1$ ● Py$_1$

5'   5'

B

Fig. 3

4/5

A

B
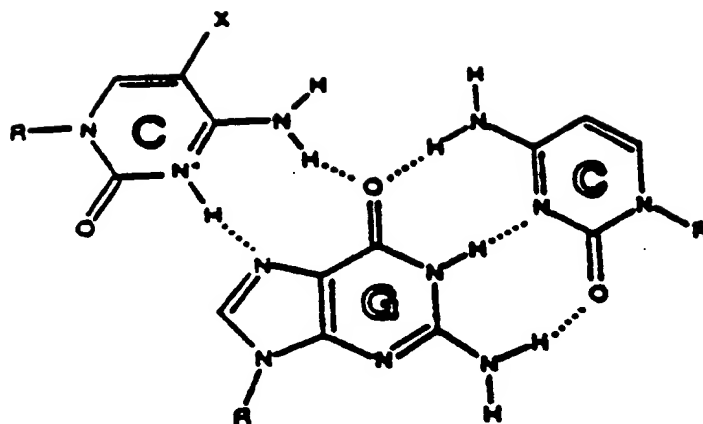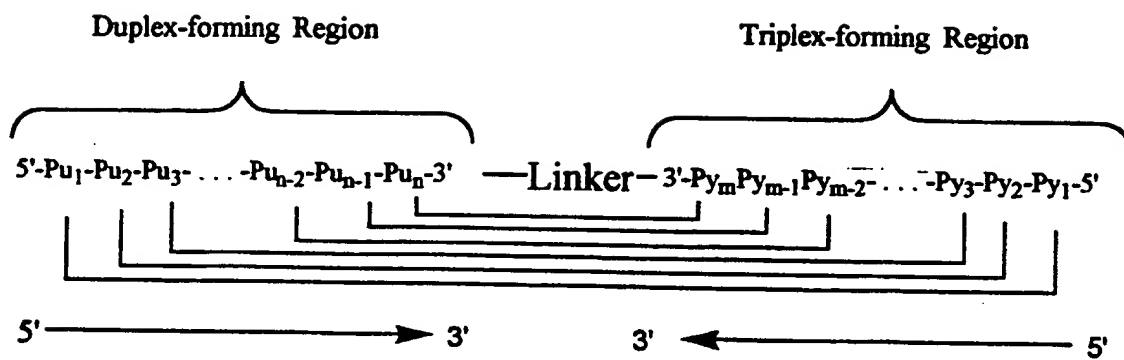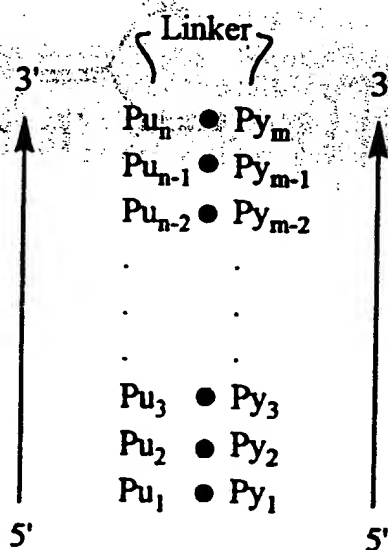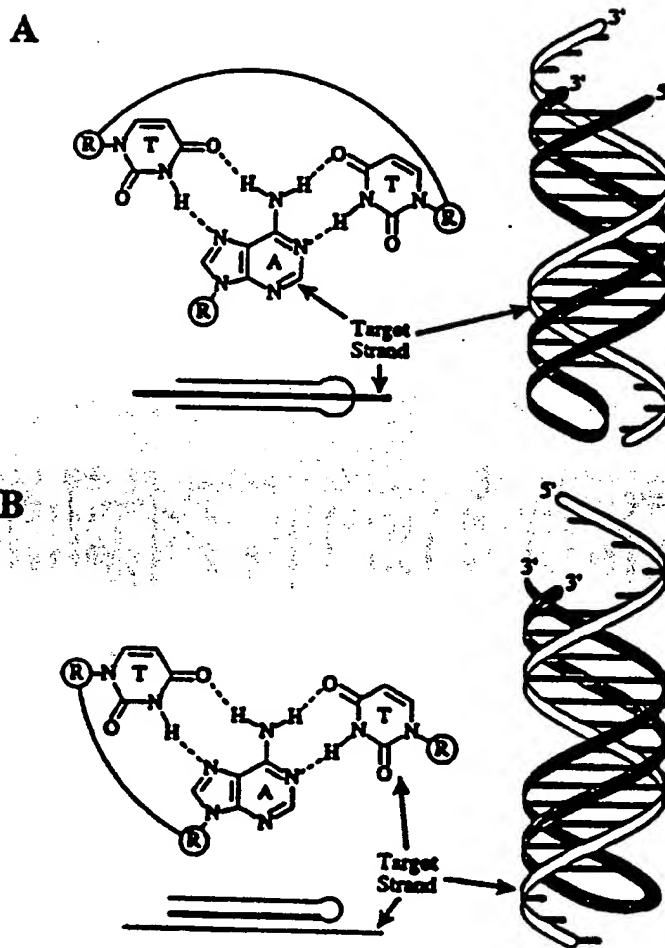
Fig. 4
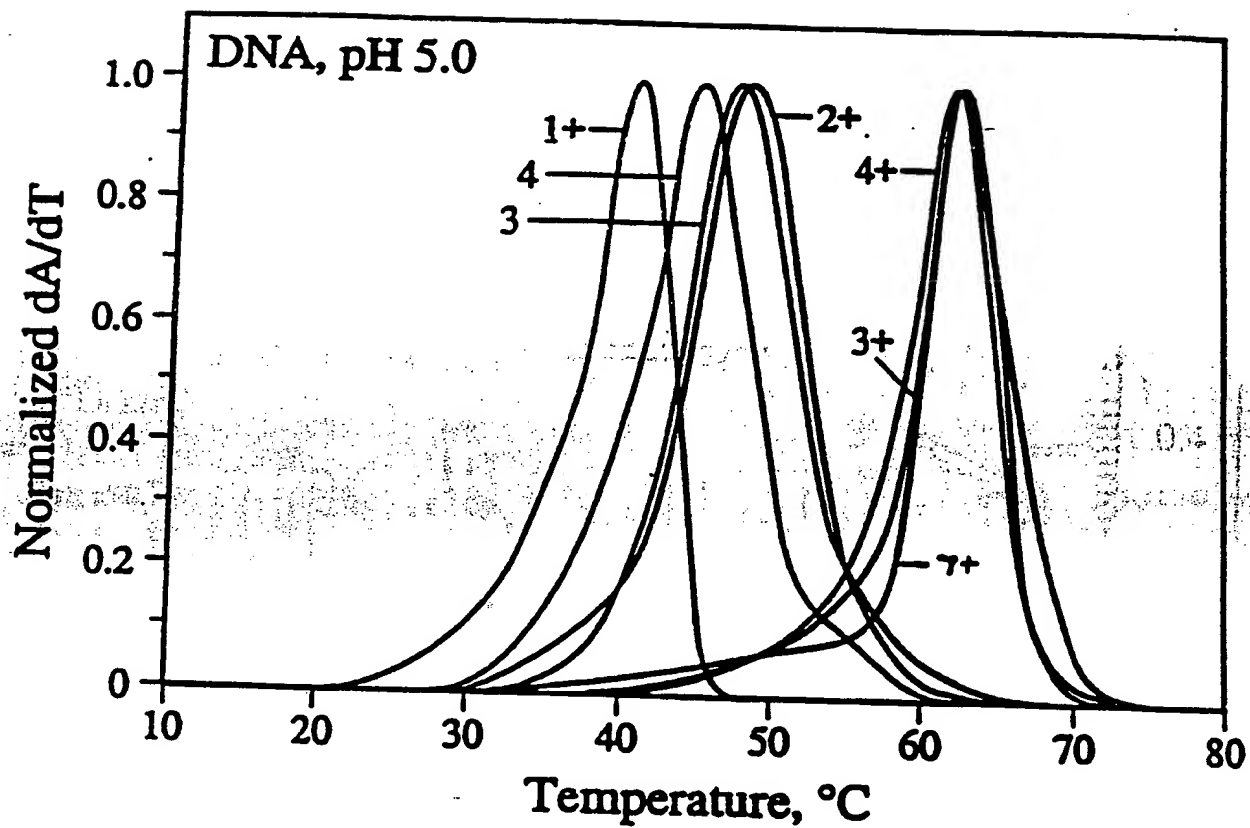
Fig. 5

International Application No

rCT/US 96/06718

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C07H21/00   C12N15/11   A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C07H   C12N   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 116, no. 19, 21 September 1994, DC US, pages 8857-8858, XP002015186 S.WANG ET AL.: "Recognition of Single-Stranded Nucleic Acids by Triplex Formation : The Binding of Pyrimidine-Rich Sequences." cited in the application see the whole document ---	1-18
A	WO,A,94 17091 (HYBRIDON INC.) 4 August 1994 see the whole document --- -/--	1,10

X	Further documents are listed in the continuation of box C.		X	Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 October 1996	2 1 -10- 1996

Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Scott, J

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOCHEMISTRY, vol. 33, no. 15, 19 April 1994, EASTON, PA US, pages 4639-4644, XP002015187 S.WANG ET AL. : "Stabilities of Nucleotide Loops Bridging the Pyrimidine Strands in DNA Pyrimidine.Purine.Pyrimidine Triplexes : Special Stability of the CTTTG Loop." see the whole document ---	1,10
A	NUCLEIC ACIDS RESEARCH, vol. 21, no. 24, 11 December 1993, OXFORD GB, pages 5616-5622, XP002015188 E.BROSSALINA ET AL.: "The Binding of an Antisense Oligonucleotide to a Hairpin Structure via Triplex Formation Inhibits Chemical and Biological Reactions." see the whole document ---	1,10
A	BIOCHEMISTRY, vol. 30, no. 18, 7 May 1991, EASTON, PA US, pages 4472-4479, XP002015189 F-M.CHEN: "Intramolecular Triplex Formation of the Purine.Purine.Pyrimidine Type." see the whole document ---	1,10
A	EP,A,0 601 889 (MAINE MEDICAL CENTER RESEARCH INSTITUTE) 15 June 1994 see abstract ---	1,10
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P,A	NUCLEIC ACIDS RESEARCH, vol. 23, no. 15, 11 August 1995, OXFORD GB, pages 2937-2944, XP002015191 T.VO ET AL.: "Targeting Pyrimidine Single Strands by Triplex Formation : Structural Optimization of Binding." see the whole document -----	1,10

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INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

> Remark : Although claims 10-18 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		CN-A-	1122138	08-05-96
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